Genetic analysis suggests a wide regional provenance distribution for *Epacris impressa* (Ericaceae)

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Abstract

Epacris impressa has showy flowers that fall into three general colour races: red, pink and white. It is primarily an outcrossing species with some examples of selfing occurring in each population. Genetic fingerprinting techniques were used to examine relationships between geographic sites and flower colour populations and to aid provenance determination. Results indicated that *E. impressa* has a high level of genetic diversity between and amongst sites and floral colour races. This suggests a wide provenance distribution for the species which would concur with earlier morphological studies conducted in the 1970's.

Key words: ISSR, RAPDs, provenance, Ericaceae, heathland

Muelleria 30(2): 175–182 (2012)

Introduction

Epacris impressa Labill. is a woody heathland shrub common to parts of south-eastern Australia. It is the floral emblem of Victoria and its range covers most of Victoria, parts of South Australia, Tasmania and southern New South Wales. Until recently, the strategy of 'local is best' has traditionally been accepted as best-practice for the collection of germplasm for native plant restoration projects. However, current studies have introduced the use of genetic fingerprinting techniques to establish plant provenance including Random Amplified Polymorphic DNA (RAPDs), Inter Simple Sequence Repeats (ISSR) and Amplified Fragment Length Polymorphism (AFLP) (Bussell *et al.* 2006; Krauss & Koch 2004; Krauss *et al.* 2005). ISSRs and RAPDs have been used together to determine phylogenetic relationships (Awasthi *et al.* 2004; Isshiki *et al.* 2008; Iruela *et al.* 2002; Levi & Rowland 1997; Mattioni *et al.* 2002; Pharmawati *et al.* 2004) and assess genetic diversity (Awasthi *et al.* 2004; Ayres & Strong 2001; Esselman *et al.* 1999; Jain *et al.* 1999). Three collection ranges were suggested for a 22,000 km^2 area in the Sydney basin using combined genetic and morphological assessment techniques: 1) narrow collection range - an area as close to the planting site as possible; 2) intermediate collection range - extending the collection area to fragmented remnants that were once contiguous with the site; 3) regional collection range – widening the geographic area to include a larger region (Burgin *et al.* 2005; Mortlock 2000). In this study, RAPDs and ISSR were performed to determine relatedness among and within geographic sites and floral colour races of *E. impressa*.

The size of heathland ecosystems has been drastically reduced and continues to be at risk due to continued land clearing. Threats to heathland areas include property and pasture development, forestry, mining, fire control in urbanised areas and infection by *Phytophthora cinnamomi* (The State of Victoria 2002; Williams *et al.* 2001). Very little research has been

Figure 1. *E. impressa* falls into three general floral colour races: a) red (Angahook-Lorne Site A); b) pink (Cranbourne Site CA) and c) white (Cranbourne Site C).

conducted into the genetic provenance of plant species in these shrinking areas of heathland and provenance is often assigned by local anecdotal information rather than on the basis of genetic traits.

A major study by Stace and Fripp in 1977 described polymorphism in *E. impressa* (Stace & Fripp 1977a, 1977b, 1977c). The flowers of *E. impressa* fall into three general races: red (fuchsia/deep pink), pink (light pink) and white (Fig. 1). Populations of *E. impressa* are often polymorphic for flower colour with monomorphic populations occurring less frequently (Stace & Fripp 1977c). Polymorphic populations exist geographically between monomorphic populations and appear to provide a conduit for gene flow (Stace & Fripp 1977b). Soil pH has been shown not to be a determining factor in flower colour and it is possible that gene flow is influenced by vector selection (Stace & Fripp 1977b, 1977c). Flowering times of different flower colours overlap at polymorphic sites but are staggered between locations by flower colour in monomorphic populations (Stace & Fripp 1977b).

Polymorphism could be an ancient trait present in *E. impressa* or a more recent development caused by hybridization between populations (Stace & Fripp 1977a, 1977c). The consistencies between corolla colour, length, and anther colour led Stace and Fripp (1977a, 1977b) to postulate that raciation in the species may be an early indicator of species division. However, further work by Fripp (1982) concluded that *E. impressa* is primarily an outcrossing species with no disparity between seedset or viability when races were crossed. Fripp (1982) self-pollinated and cross-pollinated individuals of different races from a variety of populations over a five-year period. Morphological study of germinated plants indicated that the diverse races were not separate species (Fripp 1982). The evidence suggested that raciation was probably a long-standing trait rather than an early sign of species divergence. This correlates with later genetic research that included the genus *Epacris* in Ericaceae due to early genetic links. This research was the first to use the Inter Simple Sequence Repeat (ISSR) method with a member of the Southern Hemisphere Ericaceae.

Methods

Six research sites with remnant populations of *E. impressa* were selected based on their geographic separation and to provide examples of both polymorphic and monomorphic flower-colour demes. The research sites used for genetic studies were Angahook-Lorne State Park, Victoria (Site A) (38°37'S, 143°53'E), Braeside Park Heathland (Site B), Victoria (37°59'S, 145°08'E), two sites at the Royal Botanic Gardens Cranbourne, Victoria (sites C and CA) (38°07'S, 145°16'E and 38°08'S, 145°15'E), the Grampians National Park, Victoria (site G), Sundial track (37°10'S, 142°30'E), and a privately-owned site in Tullah, Tasmania (site T) (41°44'S, 145°37'E). No white-flowered plants were observed at Angahook-Lorne State Park during the collection period and Braeside Park Heathland was monomorphic for white-flowered plants.

Genetic fingerprinting using RAPDs and ISSR was conducted in two experiments with samples from each

of the three flower colour races (white, pink and red) found at each site. After initial primer screening, ISSRs and RAPDs were performed on a subset of red, pink and white samples from each site using primers that produced the clearest reproducible banding patterns as per the following protocols.

Cuttings consisting of 10–20 cm of new tip growth and flowers were collected from flowering *E. impressa* plants from Angahook-Lorne State Park and the RBG Cranbourne sites in September 2001, from the Grampians in November 2001 and from Braeside Park Heathland and Tullah, Tasmania in September 2002. Each sample was given an alphanumeric identification according to site (A = Angahook-Lorne State Park, B = Braeside Heathland, C and CA = Royal Botanic Gardens Cranbourne sites, G = Grampians National Park, T = Tullah, Tasmania), colour (W = White, R = Red, or P = Pink) and the plant of origin. Flower colours were further identified using the *Royal Horticultural Society Colour Chart* (c1995). Plant material collected at each site was stored at -20°C until required (Table 1). Herbarium specimens were lodged at the National Herbarium of Victoria (MEL) after DNA isolation from material.

DNA was isolated from the *E. impressa* flowers using the Qiagen DNeasy® Plant kit according to the manufacturer's instructions. Electrophoresis was performed on a Horizon®58 Life Technologies™ Gibco BRL Horizontal Gel Electrophoresis Apparatus gel tray with 1% agarose in TBE stained with ethidium bromide and run at 96 V for 30 minutes. Bands of DNA were visualised on a UV transilluminator and photographed with a Kodak Digital Science DC120 digital camera. The RAPD OPB primer set was found to be successful with the ericoid, *Vaccinium macrocarpon* Ait. (Stewart & Nilsen 1995) and was selected for screening with *E. impressa*. The Operon OPB set of 20 primers (OPB1 GTTTCGCTCC, OPB2 TGATCCCTGG, OPB3 CATCCCCCTG, OPB4 GGACTGGAGT, OPB5 TGCGCCCTTC, OPB6 TGCTCTGCCC, OPB7 GGTGACGCAG, OPB8 GTCCACACGG, OPB9 TGGGGGACTC, OPB10 CTGCTGGGAC, OPB11 GTAGACCCGT, OPB12 CCTTGACGCA, OPB13 TTCCCCCGCT, OPB14 TCCGCTCTGG, OPB15 GGAGGGTGTT, OPB16 TTTGCCCGGA, OPB17 AGGGAACGAG, OPB18 CCACAGCAGT OPB19 ACCCCCGAAG) was screened initially with two DNA samples, one a red-flowered plant from Angahook-Lorne State Park (site A) and the other a white-flowered plant from Cranbourne (site C), using the protocol outlined below. Each sample and primer was run twice to test for reproducibility. Bands were obtained for both samples with the RAPD primers OPB1, OPB3, OPB4, OPB5, OPB6, OPB7, OPB15, and OPB19. From these, the two primers that yielded the clearest reproducible bands, OPB6 and OPB19, were chosen for RAPD experiments

Each RAPD/PCR reaction consisted of the following: 3.2 µl 1.25 mM dNTP, 0.8 µl 25 mM $MgCl_2$, 2 µl Tris-HCl reaction buffer, 12 µl milli-q H_2O, 1 µl Operon OPB series sequence primer, and 0.5 µl QIAGEN *Taq* DNA Polymerase. The RAPD reactions were run in a Biometra® Personal Cycler top-heating thermocycler using a 4–minute strand separation cycle at 94°C, then 45 cycles of one minute at 94°C, two minutes at 36°C, and two minutes at 72°C and a final 72°C extension step for 10 minutes. A negative control with no DNA was included in each run to test for contamination. Gels were run as previously described.

Six standard ISSR primers, 812 (GAG AGA GAG AGA GAG AA), 814 (CTC TCT CTC TCT CTC TA), 824 (TCT CTC TCT CTC TCT CG), 835 (AGA GAG AGA GAG AGA GYC), 836 (AGA GAG AGA GAG AGA GYA) and 857 (ACA CAC ACA CAC ACA CYG) were screened with two DNA samples as for RAPD screening. The primers were selected based on their ability to amplify loci in a variety of plant genera and species (Casasoli *et al.* 2001; Ge *et al.* 2003; Levi & Rowland 1997; Mattioni *et al.* 2002; Nan *et al.* 2003; Pharmawati *et al.* 2004; Qiu *et al.* 2004; Wang *et al.* 2004; Xiao & Berch 1996). ISSR reactions were prepared as

Table 1. Accession numbers for herbarium vouchers. An indicative sample was submitted for each flower colour race for sites G (Grampians National Park), CA (Royal Botanic Gardens Cranbourne), and A (Angahook-Lorne State Park). All plant material was used for DNA isolation from samples collected at sites B (Braeside Heathland), C (Royal Botanic Gardens Cranbourne), and T (Tullah, Tasmania). No white-flowered plants were observed at Angahook-Lorne State Park during the collection period.

	MEL Accession numbers		
Site	**Red/Fuchsia**	**Pink**	**White**
A	2337596	2337597	-
CA	2337594	2337595	2337593
G	2337591	2337592	2337590

Table 2. Similarity matrix for the entire sample set using the Jaccard coefficient. A = Angahook-Lorne State Park, B = Braeside Heathland, C and CA = Royal Botanic Gardens Cranbourne sites, G = Grampians National Park, T = Tullah, Tasmania. The site letters are followed by a letter indicating floral colour race W (white), R (red) or P (pink) and sample number.

	AR 18	AR 19	AP 34	BW1 1	BW1 2	CW1 9	CW2 0	CR 133	CR 134	CP 101	CP 105	CP 121	CA W24	CA W27	CA W34	CAR 4	CAP 15	CAP 17	GW 118	GW 119	GW 120	GR 137	GP 110	GP 127	TW1	TW3	TR1	TR2	TP1	TP2	TP4
AR18	1.00																														
AR19	0.75	1.00																													
AP34	0.00	0.00	1.00																												
BW11	0.00	0.00	0.00	1.00																											
BW12	0.00	0.00	0.00	0.57	1.00																										
CW19	0.33	0.38	0.29	0.18	0.08	1.00																									
CW20	0.25	0.29	0.40	0.22	0.20	0.58	1.00																								
CR133	0.25	0.13	0.00	0.33	0.31	0.27	0.21	1.00																							
CR134	0.29	0.14	0.25	0.15	0.14	0.29	0.23	0.75	1.00																						
CP101	0.18	0.20	0.20	0.13	0.29	0.42	0.36	0.20	0.10	1.00																					
CP105	0.13	0.14	0.33	0.17	0.40	0.30	0.38	0.11	0.00	0.56	1.00																				
CP121	0.09	0.10	0.25	0.13	0.29	0.33	0.40	0.20	0.10	0.89	0.63	1.00																			
CAW24	0.00	0.00	0.33	0.00	0.00	0.27	0.20	0.21	0.33	0.14	0.00	0.14	1.00																		
CAW27	0.00	0.00	0.33	0.00	0.09	0.27	0.20	0.21	0.33	0.14	0.00	0.14	0.71	1.00																	
CAW34	0.00	0.00	0.33	0.00	0.10	0.18	0.10	0.23	0.36	0.00	0.00	0.00	0.57	0.83	1.00																
CAR4	0.20	0.25	0.20	0.14	0.29	0.15	0.09	0.08	0.18	0.10	0.00	0.00	0.13	0.29	0.33	1.00															
CAP15	0.20	0.20	0.50	0.25	0.25	0.29	0.33	0.67	0.33	0.50	0.60	0.57	0.00	0.00	0.00	0.00	1.00														
CAP17	0.25	0.25	0.50	0.33	0.33	0.33	0.40	0.33	0.00	0.38	0.75	0.43	0.00	0.00	0.00	0.00	0.75	1.00													
GW118	0.00	0.00	0.25	0.20	0.17	0.20	0.25	0.29	0.14	0.22	0.00	0.25	0.14	0.14	0.00	0.00	0.17	0.00	1.00												
GW119	0.00	0.00	0.25	0.20	0.17	0.20	0.25	0.29	0.14	0.22	0.00	0.25	0.14	0.14	0.00	0.00	0.17	0.00	1.00	1.00											
GW120	0.00	0.00	0.25	0.20	0.17	0.20	0.25	0.29	0.14	0.22	0.00	0.25	0.14	0.14	0.00	0.00	0.17	0.00	1.00	1.00	1.00										
GR137	0.11	0.13	0.20	0.25	0.22	0.29	0.36	0.45	0.36	0.25	0.10	0.27	0.10	0.10	0.11	0.00	0.40	0.20	0.33	0.33	0.33	1.00									
GP110	0.00	0.00	0.33	0.00	0.00	0.27	0.20	0.25	0.50	0.38	0.17	0.43	0.29	0.29	0.33	0.11	0.40	0.20	0.11	0.11	0.11	0.43	1.00								
GP127	0.00	0.00	0.33	0.00	0.00	0.08	0.09	0.25	0.50	0.22	0.00	0.25	0.29	0.29	0.33	0.11	0.17	0.00	0.25	0.25	0.25	0.25	0.50	1.00							
TW1	0.25	0.25	0.25	0.20	0.17	0.33	0.43	0.29	0.14	0.38	0.40	0.43	0.00	0.00	0.00	0.00	0.75	0.50	0.33	0.33	0.33	0.60	0.25	0.11	1.00						
TW3	0.25	0.25	0.20	0.25	0.20	0.33	0.43	0.14	0.00	0.38	0.40	0.43	0.00	0.00	0.00	0.00	0.40	0.50	0.14	0.14	0.14	0.60	0.25	0.11	0.60	1.00					
TR1	0.00	0.00	0.33	0.17	0.17	0.30	0.22	0.43	0.29	0.40	0.43	0.44	0.20	0.20	0.25	0.00	0.60	0.40	0.17	0.17	0.17	0.38	0.40	0.17	0.40	0.17	1.00				
TR2	0.00	0.00	1.00	0.17	0.17	0.18	0.10	0.43	0.29	0.27	0.25	0.30	0.20	0.20	0.25	0.00	0.60	0.40	0.40	0.40	0.40	0.38	0.40	0.40	0.40	0.17	0.67	1.00			
TP1	0.50	0.50	0.17	0.00	0.14	0.56	0.33	0.11	0.13	0.50	0.33	0.38	0.13	0.29	0.14	0.25	0.33	0.40	0.11	0.11	0.11	0.20	0.00	0.25	0.25	0.14	0.14	1.00			
TP2	0.50	0.50	0.25	0.00	0.10	0.55	0.36	0.18	0.33	0.50	0.33	0.38	0.33	0.50	0.38	0.30	0.33	0.40	0.08	0.08	0.08	0.08	0.36	0.25	0.18	0.18	0.14	0.14	0.67	1.00	
TP4	0.50	0.50	0.20	0.00	0.00	0.63	0.38	0.00	0.00	0.50	0.33	0.38	0.14	0.14	0.00	0.13	0.33	0.40	0.13	0.13	0.13	0.13	0.22	0.00	0.29	0.29	0.14	0.14	0.83	0.56	1.00

for RAPDs and run with an initial three-minute cycle at 94°C, then 45 cycles of one minute at 94°C, 45 seconds at 54°C, one minute at 72°C, then a final extension step of 10 minutes at 72°C.

Bands were obtained for both samples with ISSR primers 812, 824, 835, and 836. From these, the two primers that yielded the clearest reproducible bands, 812 and 836, were chosen for ISSR experiments as per previously described protocols. Gel photos were visually reviewed and faint ambiguous bands were removed from the data set (Williams *et al.* 1990; Zawko *et al.* 2001). All of the gel photos were reviewed with their corresponding loci information from the Kodak1D analysis program. High (1500, 2000 and 3000 base pairs) and low (100, 200, 300 base pairs) molecular weight bands were also removed from the analysis to minimise the possibility of the inclusion of nested inverted repeats (Stewart & Excoffier 1996; Stewart & Nilsen 1995). Any individuals with no bands were excluded from the data analysis. Monomorphic bands were not present in any of the RAPD or ISSR results. A total of 182 polymorphic bands were scored with four primers for 31 individuals.

The molecular weights were then converted into binary data for presence (1) or absence (0) of bands. Any samples that were missing data from more than one primer were removed and the Exeter software program NTSYSpc 2.20e was used for statistical analyses. A similarity matrix was generated from the data set using the Jaccard coefficient, a/(n-d) (Table 2). Reduced similarity matrices were created with data sub-sets by site (Table 3) and floral colour race (Table 4) to compare the average similarities between and amongst populations. A dendrogram was created using a clustering algorithm with an unweighted pair-group method, arithmetic average (UPGMA) formula to illustrate relationships between individuals in the entire sample group (Fig. 2).

Results

ISSR and RAPD analyses showed a high level of genetic variability both between and amongst geographic and race populations of *E. impressa*. Individuals did not cluster (Fig. 2) by site or flower colour (except for small clusters of 2-3 individuals from the same site and flower colour) and inter-site similarity coefficients ranged from 13-30% and inter-colour similarity coefficients from 19-31%, with intra-site coefficients similar. The results

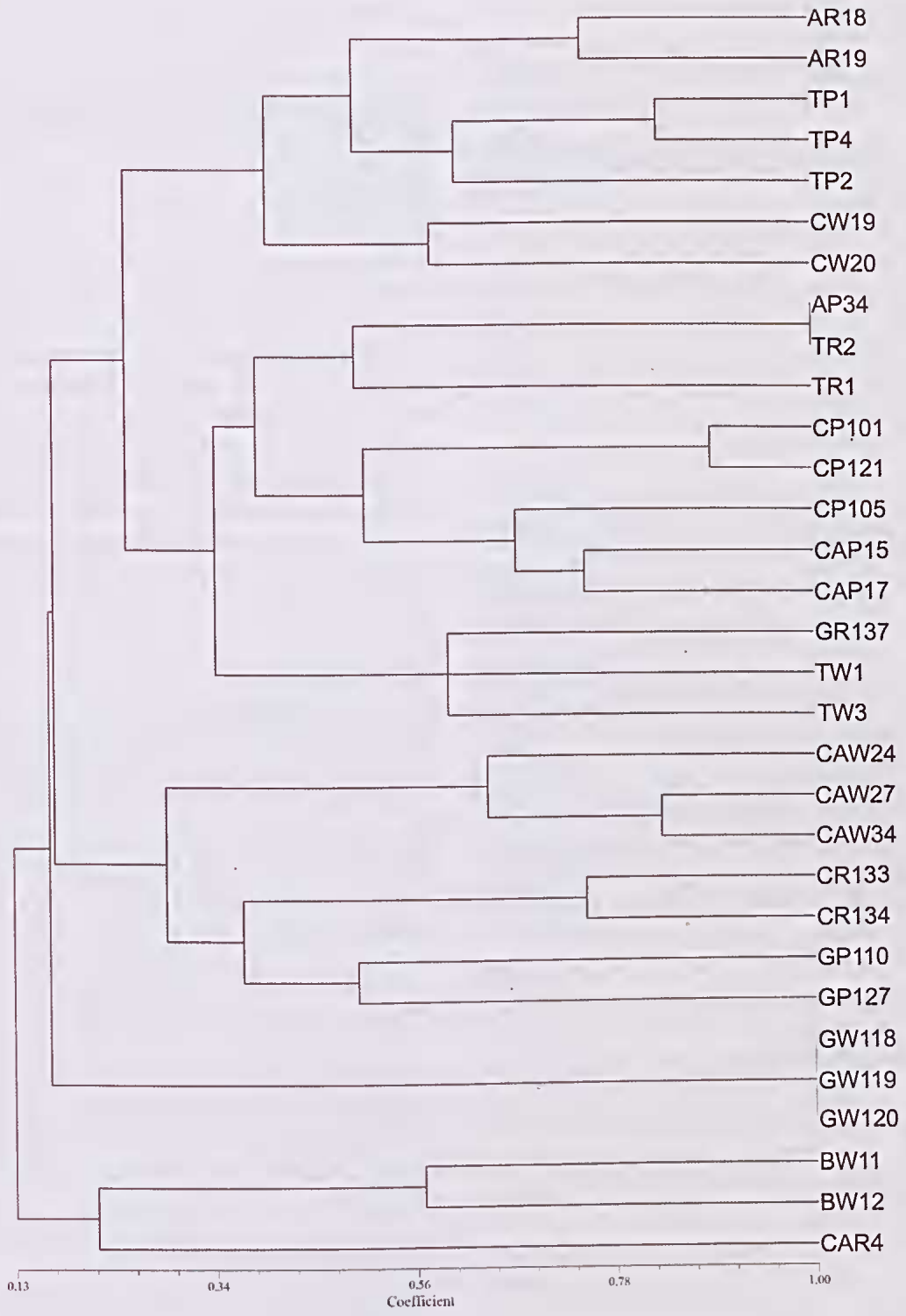

Figure 2. Dendrogram illustrating phylogenetic relationships between individuals in the entire population sample. The first letter or letters represent the collection site (A, B, C, CA, G, T) and the second letter the flower colour race (R, P, W). The number corresponds to the plant identification number.

Table 3. Reduced similarity matrix showing average percentages of similarity by site. A = Angahook-Lorne State Park, B = Braeside Heathland, C and CA = Royal Botanic Gardens Cranbourne sites, G = Grampians National Park, T = Tullah, Tasmania.

SITE	A	B	C	CA	G	T
A	0.13					
B	0.00*	0.19				
C	0.22	0.21	0.26			
CA	0.20	0.15	0.24	0.17		
G	0.10	0.13	0.23	0.14	0.30	
T	0.30	0.12	0.33	0.25	0.23	0.24

* white-flowered plants were not found at site A at the time of collection for DNA isolation.

indicated a 20% average genetic similarity between geographic populations and a 22% average similarity between colour races. The pink-flowered race had the highest average intra-population polymorphic similarity at 31%. The red and white races had the lowest inter-race and intra-race average genetic similarities (19–23%).

The percentages of similarity ranged from 10% between sites A and G to a 33% average possibility of relatedness between individuals at sites C and T (Table 3). Site A had the lowest percentage of genetic similarity (13%) of plants within its population, followed by sites CA (17%) and B (19%). Site G had the highest amount of intra-site genetic similarity (30%) followed by site C (26%) and site T (24%). There was a 20% inter-site average polymorphic similarity between plants.

Plants of the pink-flowered race had the highest average percentage (31%) of intra-race genetic similarity (Table 4). White-flowered and red-flowered plants had lower average probabilities of being genetically similar to other plants of the same flower-colour race (21% and 19% respectively). Pink-flowered plants had a 21% average genetic similarity to white-flowered plants and a 23% similarity to the red-flowered race. Red-flowered plants had an equal average probability (19%) of being related to either other red-flowered or to white-flowered plants. There was a 22% average genetic similarity between plants of any one flower-colour to those of any other race.

Clustering can be seen between individuals of the same flower colour race within populations, with the Grampians white-flowered plants (GW118–120)

Table 4. Reduced similarity matrix showing average percentages of similarity by floral colour race (W = white, R = red, P = pink).

	W	R	P
W	0.21		
R	0.19	0.19	
P	0.21	0.23	0.31

showing the highest similarity coefficient (Fig. 2). Individuals from each site are primarily clustered at low similarity coefficients or are seen grouped with members of other site populations indicating a high level of genetic variability. Clustering at the highest similarity coefficients is shown primarily by small but separate groups of red, pink, and white flowered plants. The smaller discreet colour alignments, rather than three major groupings by floral race, suggest a relatively high level of genetic variability within floral races and within sites.

Discussion

During this research and previous studies, (Stace & Fripp 1977a) plants of different flower colours in populations that were polymorphic for flower colour were observed to have separate but overlapping periods of flowering, which would have an influence on the genetic composition of the separate floral races. Native and introduced vectors that select by flower colour during overlapping periods of flowering of the three floral races would not be cross-pollinating between the floral races (Castellanos *et al.* 2003; Meléndez-Ackerman *et al.* 1997; Streisfeld & Kohn 2006). Differences in the time of flowering between flower-colours in the same population could also have an influence on pollination patterns. Research is currently being undertaken at LaTrobe University on pollinator flower-colour preference with *E. impressa* (Webster N., Edwards T. & Hoebee S. pers. comm. 2012). This may provide further insight into vector influence on flower colour composition of polymorphic populations.

The geographic isolation of the Grampians has created many unique taxa and it was not surprising that the Grampians population (site G) showed the highest level of intra-site genetic similarity (30%). A 26% average polymorphic similarity between individuals

was recorded at the Royal Botanic Gardens, Cranbourne, site C. This site primarily contained plants of the white-flowered race, which may account for the high level of intra-site genetic similarity. The Tullah site in Tasmania demonstrated a 24% average intra-site similarity. The site consisted of an area of remnant vegetation not directly geographically linked to other populations.

Angahook-Lorne site A had primarily red and pink flowered populations, and demonstrated the lowest average genetic similarity within its own population (13%). This concurs with Stace and Fripp's (1977b) findings of high levels of polymorphism for flower-colour race within linked 'mosaics' of populations. The site was also part of a large coastal range of linked *E. impressa* populations within a protected state park region. This has provided the opportunity for genetic dispersal to other geographically linked populations not sampled. Genetically, the site appears to be part of a larger population or one of a series of linked populations that share genetic traits. The recent creation of the Otways National Park will help to preserve the genetic integrity of this large polymorphic group.

Braeside site B and Cranbourne site CA also had low average intra-site polymorphic similarities at 13% and 17% respectively. These results were surprising since both sites were geographically removed from other populations. Site B comprised all white-flower race plants which could explain the polymorphic similarities. Site CA had a robust mix of races but all *E. impressa* plants at the site died during this research due to long-term drought conditions.

Epacris impressa appears to have a large regional provenance distribution. These findings concur with Stace and Fripp's (1977a; 1977b; 1977c) earlier work on raciation of the species which found high levels of polymorphism for flower colour race, corolla colour and corolla length. While geographic populations showed high levels of inter-site genetic diversity, morphological characteristics should still be considered when collecting propagative material for revegetation. Since genetic fingerprinting techniques target unknown regions of the genome, locally adaptive traits may not be represented by the loci in RAPD and ISSR analysis (O'Brien *et al.* 2007). A combination of genetic fingerprinting and traditional morphologic observation is recommended to determine provenance (Krauss *et al.* 2005) and plant propagules should still be collected from populations of similar floral colour race (Stace & Fripp 1977b). Hence, when trying to re-establish a population of all white-flowered plants, it is suggested that cutting material is collected from another site of all white-flowered plants. Geographic proximity of populations does not appear to be an issue in provenance determination based on the regional genetic spread of *E. impressa*. This has been found to be the case in other Australian studies where large regional areas of provenance have been established with DNA fingerprinting techniques (Krauss & Koch 2004; Krauss *et al.* 2005).

Based on this research, *E. impressa* has a high level of both intra-race and inter-race genetic diversity. The inter-site genetic similarities indicate that *E. impressa* populations have probably been geographically inter-connected until recently. Further work will be required on Tasmanian populations to determine their genetic links to mainland South-eastern Australia plants and additional studies in southern New South Wales and South Australia would be valuable.

Acknowledgements

Our thanks to the curatorial staff at the National Herbarium of Victoria, editors and reviewers for comments on this paper. Sincere thanks and appreciation to a dedicated researcher and mentor, Dr Cassandra McLean[1], who succumbed after a long and hard battle with cancer in 2009. Thank you to the Australian Flora Foundation for a generous grant that helped support this research.

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